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Cell culture models using rat primary alveolar type I cells

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Abstract

There is a lack of cell culture models using primary alveolar type I (AT I) cells. The purpose of this study was to develop cell culture models using rat AT I cells and microvascular endothelial cells from the lung (MVECL). Two types of model systems were developed: single and co-culture systems; additionally a 3-dimensional model system was developed. Pure AT I cell (96.3 ± 2.7%) and MVECL (97.9 ± 1.1 %) preparations were used. AT I cell morphology, mitochondrial number and distribution, actin filament arrangement and number of apoptotic cells at confluence, and telomere attrition were characterized. AT I cells maintained their morphometric characteristics through at least population doubling (PD) 35, while demonstrating telomere attrition through at least PD 100. Furthermore, AT I cells maintained the expression of their specific markers, T1 α and AQ-5, through PD 42. For the co-cultures, AT I cells were grown on the top and MVECL were grown on the bottom of fibronectin coated 24 well Transwell Fluroblok™ filter inserts. Neither cell type transmigrated the 1 micron pores. Additionally AT I cells were grown in a thick layer of Matrigel® to create a 3-dimensional model in which primary AT I cells form ring-like structures that resemble an alveolus. The development of these model systems offers the opportunities to investigate AT I cell cells and their interactions with MVECL in response to pharmacological interventions and in the processes of disease, repair and regeneration.

Keywords

Rat; Cell culture models; Alveolar type I cells; Microvascular endothelial cells; Three-dimensional culture; Alveolus

1. Introduction

The alveolus, or gas exchanging surface of the lung, is composed of alveolar epithelial type I (AT I), type II (AT II) and microvascular endothelial cells. AT I cells are large flat squamous epithelial cells that cover 95-99% of the alveolar surface area and participate in gas, ion and water exchange. AT II cells are cuboidal shaped cells that produce surfactant, are more numerous than AT I cells, and account for only ~1% of the alveolar surface area [1-3]. The alveolar epithelium and endothelium share a common basement membrane which reduces the distance gases must diffuse from the alveolar space to the vasculature and vice

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versa. However in the setting of many disease states, such as emphysema and acute respiratory distress syndrome, the alveolus is damaged leading to hypoxia and hypercapnia, or elevated carbon dioxide.

Our understanding of the roles that AT I cells play in the development and in the response to treatments for lung disease is hindered by a lack of cell culture model systems using primary AT I cells. The predominant reason is that primary AT I cells have been considered terminally differentiated—that is, incapable of mitosis—and difficult to isolate and maintain in culture. As a result AT I cells differentiated in vitro from alveolar type II cells, termed AT I-like cells, are used as a surrogate [4]. However the similarities and differences of AT I cells and AT I-like cells are not fully understood. The purpose of this paper is to describe the development of model systems using rat primary AT I and microvascular endothelial cells from the lung (MVECL).

In our studies, we find that rat primary AT I cells undergo mitosis while maintaining the expression of AT I specific markers, T1 α (RT140, rat podoplanin)[5] and aquaporin-5 (AQ-5) [6], through at least population doubling (PD) 42. Furthermore, we describe the development of a co-culture model in which rat primary AT I cells express T1 α , AQ-5, and zona occludin-1 (ZO-1) when grown on the top, and MVECL express VE-cadherin when grown on the bottom of fibronectin coated 24-well Transwell Fluroblok™ filters. In addition, when grown in a 3-dimensional matrix, rat primary AT I cells form ring-like structures that resemble an alveolus. These model systems offer opportunities to investigate the structural and functional properties of AT I cells, while the establishment of a co-culture model system allows for the study of interactions between AT I cells and MVECL in response to pharmacological interventions and in the process of disease, repair and regeneration.

2. Materials and Methods

2.1 Materials

2.1.1 Animals—Pathogen-free 3 month old male Fischer 344 rats (~280 grams) were obtained from Harlan Laboratories (Indianapolis, IN) and used as the source of primary AT I cells and MVECL. Protocols for animal use were approved by the Institutional Animal Care and Use Committees of The University of Arizona and The Southern Arizona VA HealthCare System (SAVAHCS).

2.1.2 Reagents and assay kits—Unless stated otherwise, all laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Vybrant® CFDA SE cell tracer kit, Vybrant® DiI cell tracer, 4',6-diamidino-2-phenylindole (DAPI), rhodamine-conjugated phalloidin, Dynabeads® M-280 sheep anti-rabbit IgG, the CELLlection™ Biotin Binder Kit, and DNA primers were purchased from Invitrogen (Carlsbad, CA). The CellTiter® AQ_{ueous} One Solution Assay, an MTS assay, was purchased from Promega (Madison, WI). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Elastase was purchased from Worthington (Lakewood, NJ). Matrigel was purchased from BD Lifesciences (Franklin Lakes, NJ). The mitochondria staining kit, modified papanicolaou staining kit, alkaline phosphatase staining kit, leukocyte esterase staining kit, and RPMI 1640 media were purchased from Sigma-Aldrich (St. Louis, MO). EGM-2MV media were purchased from Lonza (Basel, Switzerland). The Qiagen QIAamp DNA Blood Midi kit was purchased from Qiagen (Valencia, CA). Brilliant SYBR green qPCR reagent kit was purchased from Stratagene (Cedar Creek, TX).

2.1.3 Antibodies—Rabbit anti-rat Aquaporin-5 (Product No. sc-28628), rabbit anti-rat PECAM-1 (Product No. sc-28188) and rabbit anti-rat anti-von Willebrand factor (Product

No.sc-14014) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-rat ZO-1 (Product No. 402200) and Alexa Fluor® 568 donkey anti-goat IgG secondary antibody (Product No. A11057) were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-rat podoplanin (Product No. P2120) and rabbit anti-human VE-cadherin (Product No. V1514) and goat anti-vimentin (Product No. V4630) were purchased from Sigma-Aldrich (St. Louis, MO). Rhodamine (TRITC) conjugated affinity purified goat anti-rabbit IgG secondary antibody was purchased from Jackson ImmunoResearch (Product No. 111-025-003; West Grove, PA).

2.2.1 Cell isolation and culture—AT I cells were obtained from the lungs of pathogen-free 3 month old male Fischer 344 rats (~280 grams) using a previously published protocol [7]. In brief, rats were anesthetized with isoflurane and the lungs extracted. The trachea was cannulated and the lungs lavaged with PBS containing 5 mM EDTA and 5 mM EGTA. The lungs were lavaged three times with RPMI 1640 with 10% FBS, 10% Dextran 40, and 4.5 U/mL elastase for ten minutes at 37° C. After the trachea and large airways were removed, the lung tissue was minced into approximately 1 mm³ pieces and mechanically ground (using a syringe plunger) through 160 and 37 micron filter mesh to produce a cell suspension. Following centrifugation (250 g for 8 minutes) the cell suspension was resuspended in 0.5 mL of RPMI 1640 with 1% FBS, 25 mM HEPES, and 100 µg/mL DNase I. Dynabeads® M-280 sheep anti-rabbit IgG coated magnetic beads were incubated with 1 µg rabbit anti-rat podoplanin antibody for 30 minutes at 4°C. Following conjugation, the beads were incubated with the cell suspension for 45 minutes at 4°C with end-to-end rotation and were used to positively select for AT I cells. The MVECL were isolated using a previously published protocol[8] in which cells were positively selected by using the CELlection™ Biotin Binder Kit, a magnetic bead kit. AT I and MVECL were grown separately in 25 cm² tissue culture flasks and maintained in RPMI 1640 with 10% FBS (AT I) or EGM-2MV (MVECL) medium. Human dermal fibroblasts derived from normal neonatal foreskin, a gift from Dr. Steven Goldman at SAVAHCS, were grown in RPMI 1640 media with 10% FBS and used as a positive control for vimentin staining as discussed below.

2.2.2 Immunocytochemical markers and microscopy—Immunocytochemical staining was performed to assess the purity of freshly isolated cell preparations and to evaluate maintenance of T1α and AQ-5 expression at successively higher PDs. To assess cell purity, freshly isolated AT I and MVECL cell preparations were grown on gelatin-coated 8-well Permanox chamber slides (VWR) for 48 hours. Then samples were washed with PBS without calcium and magnesium, fixed with 4 % paraformaldehyde in PBS for 5 minutes, and then washed 5 times with PBS. Samples to be stained for AQ-5, VE-cadherin and von Willebrand factor were permeabilized for 2 minutes with 0.5% Triton X-100 in PBS. To prevent non-specific binding, the samples were blocked with PBSAA (PBS with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide) containing 10% goat serum for 15 minutes at 37°C. The respective primary antibodies diluted in PBSAA with 0.05% Tween-20, 10% goat serum and 1% BSA were added and incubated at 37°C for 1 hour. The samples were washed, and the secondary antibody in PBSAA with 0.05% Tween-20 was added and incubated at 37°C for 1 hour. Then the samples were washed, mounted using Vectashield, and images taken using a SPOT™ camera (Diagnostic Instruments) attached to an inverted microscope (Leica DMIRB) with brightfield, phase contrast, differential interference contrast (DIC), and fluorescence optics. Image analysis was performed using either Image-Pro-Plus™ Software (Media Cybernetics) or Image J software (NIH).

To assess expression of T1α and AQ-5 at successively higher PD (6, 17, 30 and 42), AT I cells (2.0×10^4) were seeded on gelatinized 8-well Permanox chamber slides and grown in RPMI 1640 with 10% FBS. The samples were incubated overnight at 37° C in 5% CO₂ then

fixed with 4% paraformaldehyde, and immunocytochemical staining for T1 α and AQ-5 was performed as described above. Fifteen images of cells at each PD were taken and used to quantify fluorescence intensity and percent of cells positive for T1 α and AQ-5. Percent of positive cells was calculated as the percentage of cells having a measured fluorescence value of ≥ 2.5 standard deviations above the control. Samples stained with secondary antibody only were used as negative controls for each marker.

Potential contaminants in AT I cells preparations included AT II cells, macrophages, and alveolar fibroblasts. To quantify contamination in freshly isolated AT I cell preparations, cells from each AT I cell preparation were grown on gelatin coated 8-well glass chamber slides for 48 hours in RPMI 1640 with 10% FBS. Then the modified papanicolaou staining kit [9] and alkaline phosphatase staining kit [10] were used to detect AT II cells and a leukocyte esterase staining kit was used to detect macrophages [10]. Cell preparations were stained to detect vimentin, a marker for mesenchymal cells; human dermal foreskin fibroblasts were used as a positive control. Digital images of stained cells were captured and used to quantify AT II cells, macrophages, and fibroblasts.

2.2.3 AT I cell characterization—To characterize rat primary AT I cells we first evaluated AT I cell morphology. Four randomly selected microscopic fields (1 mm²) of confluent AT I cells (Figure 2, C) at PD 6-7, 20-24, and 30-35 were digitally captured and analyzed. The following morphological characteristics for cells were determined for each 1 mm² microscopic field: 1. cell number, 2. cell area (μm^2) for 20 randomly selected cells, 3. number of “senescent cells” (defined as two or more nuclei)[11, 12], and 4. “senescent” cell area (μm^2). Senescence-associated β -galactosidase was not a reliable measure of senescence in AT I cells due to elevated enzyme activity across all PDs (data not shown). To determine actin filament orientation in AT I cells, 1.0×10^5 AT I cells were grown on gelatinized 8-well chamber slides to confluence. The cells were fixed with 4% paraformaldehyde, permeabilized for 5 minutes with 0.05% Triton X-100, and stained with phalloidin conjugated to rhodamine. Cells were examined microscopically, and digital images captured.

Second, the mitochondrial staining kit was used to determine the number and distribution of mitochondria in AT I cells. Cultured AT I cells at PD 6-7, 20-24 and 30-35 were grown to confluence on gelatinized 8-well chamber slides and then labeled with the mitochondrial staining kit dye (JC-1) according to the manufacturer’s instructions. Next digital images of the stained cells were captured for analysis. Computer software was used to create an area of interest (10 μm^2) which was used to determine the number of mitochondria per 10 μm^2 adjacent to the nucleus and 10 μm^2 away from the nucleus.

Third, the CellTiter® AQ_{ueous} One Solution was used to evaluate relative AT I cell number. Rat primary AT I cells (1.0×10^3 cells/well) were grown on four gelatinized 96-well plates in RPMI 1640 with 10 % FBS and medium changed every 48 hours. AT I cells were incubated at 37°C in 5% CO₂ for 1 hour with CellTiter® AQ_{ueous} One Solution and the reaction stopped with 10% SDS in PBS. Absorbance was read at 492 nm using a Tecan GENios plate reader at 24, 48, 72 and 96 hours (one plate per time point).

Fourth, the mitochondrial staining kit was used to determine the number of apoptotic AT I cells. Cultured AT I cells at PD 6-7, 20-24 and 30-35 were grown to confluence on gelatinized 8-well chamber slides and then labeled with the mitochondrial staining kit dye, JC-1. The JC-1 dye forms red fluorescent aggregates in intact mitochondria that turn green when the mitochondrial membrane is disrupted, an indicator of apoptosis. Digital images of the stained cells were captured and analyzed.

Last, to determine relative telomere length, DNA was extracted from AT I cells at PD 6-7, 21-23, 30-35 and 100 using the Qiagen QIAamp DNA Blood Midi kit per the manufacturer's instructions. Cawthon's method for determining relative telomere length using quantitative real-time polymerase chain reaction (qRT PCR) was used[13]. This method determines relative telomere length by obtaining a ratio of cycle to threshold for telomeres relative to a single copy gene for each cell sample.

2.2.4 Co-culture on Transwell Fluroblok™ filter inserts—MVECL (2.0×10^5) were seeded on the bottom of inverted fibronectin-coated (50 $\mu\text{g/mL}$) 24-well Transwell Fluroblok™ filter inserts with 1 micron pores (BD Lifesciences, Franklin Lakes, NJ) and incubated with RPMI 1640 with 10% FBS medium for 2 h at 37°C under 5% CO₂. The filter inserts were then inverted and placed in 24-well plates filled with 600 μL per well of RPMI 1640 medium with 10 % FBS; AT I cells (1.0×10^5) were seeded on the top surface of the filter inserts and grown to confluence simultaneously with the MVECL. The next day the medium was changed to RPMI 1640 with 20% FBS and replaced every second day. Co-cultures were maintained at 37°C under 5% CO₂ for 4 days.

To assess for cell transmigration through the filter, AT I cells were labeled with DiI, a hydrophobic cyanine dye (fluoresces red) and MVECL with CFDA SE (fluoresces green). The labeled cells were seeded on opposite sides of the filter as described above and allowed to grow for 48 hours, and then the filter was excised, mounted on a coverslip with Vectashield, and digital images of cells on each side captured.

2.2.5 Electron microscopy—For transmission electron microscopy, co-cultures grown on filters were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for 30 minutes, then fixed in 1% OsO₄ for 2 hours and dehydrated in a graded ethanol series. The filters with cells were embedded in epoxy resin and polymerized at 60° C for 48 hours. Ultra-thin sections were then cut perpendicular to the filter surface with an ultramicrotome and collected on copper grids. After the addition of uranyl acetate, sections were viewed and images taken using a transmission electron microscope (model CM-12; Philips).

2.2.6 Primary AT I cells in two and three dimensional culture—To produce a 2-dimensional culture, primary AT I cells at PD 6 (1.0×10^3) were grown on individual wells of glass chamber slides coated with a thin layer (10 μL /well) of Matrigel. Cells were maintained in RPMI 1640 with 10 % FBS for 14 days; medium was changed every two days. A 3-dimensional culture of AT I cells was created by growing primary AT I cells at PD 6 (1.0×10^3) into a thick layer (50 μL /well) of Matrigel in individual wells of an 8-well glass chamber slide. Cells were maintained in RPMI 1640 with 10% FBS for 15 days; medium was changed every two days. Staining of nuclei was performed by incubating diluted DAPI (5 μL DAPI in 5 mL RPMI 1640 with 10% FBS) in sample wells for 10 minutes; cells were maintained in fresh media following DAPI incubation. To produce a 3-dimensional co-culture, AT I cells and MVECL at PD 6 (1.0×10^3) were mixed together in RPMI 1640 and seeded on a thick layer of Matrigel and maintained in RPMI 1640 with 10% FBS for 14 days. Images were captured using phase contrast, DIC and fluorescence microscopy.

2.3 Statistical Analysis

Single comparisons were performed using the student's t-test. Multiple comparisons were performed using one way analysis of variance (ANOVA) followed by Dunn's Method for all post hoc pairwise comparisons. Probability values of less than 0.05 were considered significant. Data presented below are expressed as means $\pm$ SEM.

3. Results

3.1 Purity of cell preparations and AT I cell characteristics

Purity of AT I cell preparations was $96.3 \pm 2.7\%$ as determined by immunocytochemistry to detect T1 α (See Figure 1, A) and AQ-5 (See Figure 1, B). Potential contaminants included AT II cells, macrophages and fibroblasts. There was minimal ($\sim 1\%$) contamination with AT II cells and macrophages. Vimentin staining was performed to detect alveolar fibroblasts (See Figure 1, C-E) and $< 1\%$ of cells in the AT I cell preparation exhibited vimentin. Vimentin was observed in MVECLs (data not shown) and in positive control samples using human dermal foreskin fibroblasts (See insert Figure 1, C). Purity of MVECL cell preparations was $97.9 \pm 1.1\%$ as determined by immunocytochemistry to detect VE-cadherin (Figure 1, F) and von Willebrand factor (data not shown).

While characterizing freshly isolated AT I cells, we observed an increase in AT I cell number. To further describe our observation, AT I cells were grown on gelatinized slides and digital images captured for 8 days (Figure 2, A-C). One day post seeding the AT I cells had adhered to the gelatinized slide and begun to spread (Figure 2, A). By day 4, AT I cells had increased in cell number (Figure 2, B), and by day 8, AT I cells had formed a monolayer (Figure 2, C). Freshly isolated AT I cells continued to propagate to very high population doublings (PD)—at least to PD 135. To quantify AT I cell proliferation, the Cell Titer 96[®] AQueous One Solution Cell Proliferation Assay, an assay used to quantify relative viable cell number was performed. AT I cells demonstrated a two- fold increase in relative cell number by 96 hours (Figure 2, D). Therefore qualitative and quantitative data confirm that AT I cells proliferate well in vitro.

AT I cell morphometric characteristics remained stable through at least PD 35 (Table 1). Furthermore, statistical data revealed that AT I cells at PD 6-7, 20-24 and 30-35 did not differ based on cell area, cell perimeter and senescent cell area. In addition, staining of mitochondria allowed for the determination of mitochondrial number, their distribution and the quantification of apoptotic cells. The number of mitochondria ranged from 2.22-2.57 per $10 \mu\text{m}^2$ with a many of the mitochondria surrounding the nucleus (Figure 3, A)—e.g. in a peri-nuclear fashion. AT I cells at PD 6-7, 20-24 and 30-35 demonstrated minimal apoptosis ($\sim 1\%$), and peripheral bands of actin were observed in confluent AT I cells (Figure 3, B).

AT I cells demonstrate telomere loss through at least PD 100 with approximately 87 base pairs being lost per cell division (Figure 3, C). Despite the ability to proliferate to high PD number, telomere shortening occurred, indicating that the AT I cells had not become immortal.

AT I cells at PD 6, 17, 30 and 42 were evaluated to determine if the expression of T1 α and AQ-5 was stable (Figure 4, A-H). Statistical analysis revealed that the fluorescence intensity of T1 α and AQ-5 stained cells (Figure 4, I) and the percentage of AT I cells expressing T1 α and AQ-5 (Figure 4, J) remained unchanged through at least PD 42, a finding not previously reported.

Our observations of primary AT I cells confirm that they proliferate, maintain their morphometric characteristics, and demonstrate long-term proliferative capacity with telomere attrition while maintaining the expression of AT I specific markers. These observations make primary AT I cells appealing for cell culture models.

3.2 Model systems using rat primary AT I cells

Rat primary AT I cells and MVECL were used in the development of model systems. In the co-culture model, AT I cells were grown on the top and MVECL grown on the bottom of

fibronectin coated filters. To establish a co-culture model, we first evaluated for transmigration by seeding fluorescently labeled AT I and MVECL on opposite sides of the filter and capturing images (Figure 5, A and B). Neither cell type transmigrated through the 1 micron pores indicating each cell type remained on the surface to which it was seeded, thereby creating compartments composed of a single cell type.

Second, electron microscopy was performed to determine if AT I cells retain their morphology when grown on the filter and in co-culture with MVECL. AT I cells grown on the filter possess microvilli, mitochondria, caveolae, a basement membrane and form tight junctions (Figure 5, C and D). Others have reported similar findings of AT I cells grown on chamber slides [4, 14].

However, in 5% of the samples grown on the fibronectin coated filters, we observed that the AT I cells developed a more square or rectangular shape (See Figure 5, B) and did not form a discreet monolayer. However, this is the first study to report that AT I cells grown in co-culture with MVECL retain many of their morphometric characteristics.

Third, AT I cells were evaluated to determine if they express T1 α , AQ-5 and ZO-1, a tight junction protein, when grown on the fibronectin coated filter surface and in co-culture with MVECL. Figure 6 A-C shows the presence of T1 α , AQ-5 and ZO-1 in AT I cells grown on the filter. Interestingly, we observed that AQ-5 localized to the cell periphery, the cytoplasm and the nucleus, a finding not previously reported. Control wells with secondary antibody only did not demonstrate nuclear staining suggesting alternate locations of AQ-5 other than the plasma membrane. Although peripheral bands of ZO-1 were observed, the nucleus was more strongly stained. Furthermore, there was co-localization of ZO-1 with DAPI, as evidenced by the purplish-pink DAPI coloring, indicating a nuclear location for ZO-1. VE-cadherin, an endothelial cell marker, was observed in monolayers of MVECL grown on the filter (Figure 6, D). The presence of these markers is significant; it suggests AT I cells and MVECL continue to express their in vivo markers when grown as a co-culture.

To create a 3-dimensional culture, primary AT I cells at PD 6 were grown in a thick layer (50 μ L) of Matrigel[®] and images captured daily for 14 days. We also grew AT I cells on a thin layer (10 μ L) of Matrigel[®] and found similarities to AT I cells grown on gelatin (Figure 7, A). In the 3-dimensional culture, rat primary AT I cells remained rounded for the first 24 hours, but by day 2 the AT I cells had begun to form a network of elongated cells (Figure 7, B). By day 8 the elongated cells appeared to form complex lattice-like networks (Figure 7, C), and by day 11, a few ring-like structures (Figure 7, D) began to develop. On day 15 the viability of the cultures significantly diminished as one-half of the cultures died. Despite the identical composition of the extracellular matrix, the development of ring-like structures depended upon AT I cells being grown in a thick layer of the Matrigel[®].

The size and number of the ring-like structures observed from AT I cells grown in thick Matrigel[®] varied, although the overall incidence per microscopic field was between 5 and 12% (cells per field) with an average diameter of ~50-55 μ m (Figure 7, D) which is similar to the size of rat alveoli in vivo [15]. In addition, the ring-like structures were composed of at least 2 cells as determined by DAPI staining of nuclei (Figure 7, D insert). Since not observing ring-like structures in cultures grown on thin Matrigel[®], our results suggest that the formation of ring-like structures is dependent upon AT I cells being grown in a thick layer 3-dimensional matrix. In addition, after mixing AT I cells and MVECL cells together, the combined cell suspension was plated and grown on 3-dimensional cultures with RPMI 1640 with 10% FBS. Under these conditions, we observed that AT I cells developed lattice-like networks while MVECL formed small (< 5 μ m in diameter) ball-like structures (data not

shown). However, no discernable structural connection between AT I cells and MVECL formed.

4. Discussion

In the current study we find that rat primary AT I cells proliferate to very high population doublings with evidence of telomere shortening. In addition, AT I cells maintain the expression of T1 α and AQ-5 through at least population doubling 42. The ability to isolate and propagate AT I cells, while they maintain the expression of their markers, makes them appealing for the development of cell culture models as described here. Two types of model systems were developed: single culture models and a co-culture model.

A few additional points warrant further discussion. First, this is the first study to describe both the ability of AT I cells to proliferate to very high population doublings and to demonstrate evidence of telomere shortening. Gonzalez et al (2009) previously reported that AT I cells proliferated up to 16 days in culture [16]; however, we describe the ability of primary AT I cells to proliferate to at least to PD 135. These findings suggest that the commonly accepted belief that AT I cells are incapable of cell division warrants reconsideration. This belief is based on studies of Evans et al [17] and Adamson and Bowden [18] who describe the differentiation of AT II cells into AT I-like cells following oxidant injury of AT I cells. This led to the concept that AT I cells were ‘terminally differentiated’ and incapable of mitosis. Our data clearly demonstrate that AT I cells are capable of mitosis in vitro which raises concerns about the validity of the conclusion that AT I cells are incapable of proliferation. However, additional study is needed to determine the similarities and differences in proliferative ability of AT I cells in vivo and in vitro.

Second, it is important to note that telomere shortening occurred in proliferating AT I cells, and that proliferating AT I cells maintained their morphometric characteristics and the expression of T1 α and AQ-5. Telomere shortening does not occur in immortalized cells, such as cancer cells, because cancer cells typically express telomerase which replaces telomeric DNA that is lost during replication [19]. Hence this suggests that AT I cells had not become immortal. The ability of AT I cells to maintain their markers, as well as their morphometric characteristics, suggest that proliferating AT I cells maintain many of their phenotypic characteristics in vitro. In addition, we have had success in freezing and resurrecting rat primary AT I cells which affords the opportunity to bank AT I cells and minimizes the number of animals needed to procure them. To our knowledge this is the first study to report these findings of primary proliferating AT I cells, which collectively, allow for the use of primary AT I cells in the development of cell culture models that can be used for larger scale studies.

Third, AT I cells, like many other cell types from a variety of mammalian sources [20, 21], are believed to exhibit phenotypic plasticity when grown on different extracellular matrices [16]. In our hands, rat primary AT I cells occasionally (approximately 5% of filter samples) exhibited a different morphology when grown on the Transwell filter—that is, the AT I cells would assume a square or rectangular shape (See Figure 6, B). However, despite an alteration in cell morphology, the AT I cells expressed their markers when grown on the fibronectin coated filters. It is commonly believed that aquaporin 5 is localized to the plasma membrane. However, we observed nuclear and cytosolic localization of AQ-5 in cultures of primary AT I cells. Nuclear staining of aquaporin proteins has been reported in a variety of cell types. For example, nuclear staining of aquaporin 0 has been described in the rat retina [22] and aquaporin 1 in corneal endothelial cells [23]. Furthermore, the translocation of the subcellular localization of aquaporin-5 in the rat parotid gland has been observed following chemical induction with muscarinic drug [24]. Last, it is important to recognize that

published data from aquaporin 5 staining is typically of tissue sections. Our data are much different in that our staining of aquaporin-5 is of freshly isolated AT I cell preparations. In addition, the expression of ZO-1 was observed in the cell periphery as well as colocalizing with DAPI in the cell nucleus. Nuclear localization of ZO-1 is common in proliferating cells and has been extensively described in the gut epithelium during shedding or following injury [25].

Fourth, we describe the establishment of two types of model systems: single culture models and a co-culture model. The co-culture model is composed of rat primary AT I and MVECL grown on opposite sides of a filter where each cell type retained many of their in vivo characteristics and without evidence of transmigration. Previously, AT I-like cells, derived from AT II cells, have been used as a surrogate for primary AT I cells in the development of other co-culture model systems because of perceived difficulties with the isolation and propagation of primary AT I cells [1, 10, 18]. It is not fully understood if AT I-like cells completely mimic the biochemical and functional properties of AT I cells. The cell culture models described here offer opportunities to study primary AT I cells instead of AT I like cells.

The 3-dimensional culture developed here is composed of rat primary AT I cells. More complex 3-dimensional cultures composed of multiple cell types have been extensively used in breast cancer research in which studies have demonstrated that breast cancer cells, from numerous breast cancer cell lines, maintain distinctive morphologies as well as reproducible gene expression changes [26-30]. In addition, key microenvironmental cues underlying structural and functional properties of the mammary epithelium have been identified, and from these studies it is known that the composition of the extracellular matrix is necessary to induce polarity and acinar morphogenesis [31]. To date, only AT II cells from rabbit neonates have been used in 3-dimensional cultures and were observed to form ‘alveolar-like’ structures [9]. Despite seeding a single cell type, the current 3-dimensional culture system described in this study may be useful for future investigations aimed at understanding how AT I cells respond to key biochemical cues. More specifically, the current 3-dimensional culture system could be used to determine how AT I cells respond to a variety of stimuli such as cigarette smoke, pharmacological agents or lipopolysaccharides. In addition, the 3-dimensional model may be useful for the manipulation of biochemical cues that regulate the structure of AT I cells.

In the current study we describe the development of cell culture models using rat primary AT I cells. Two types of model systems were developed: single and co-culture model systems. In addition, a 3-dimensional model using primary AT I cells was developed. The model systems using primary AT I cells, as described here, could be used to investigate AT I cells responses to a variety of stimuli such as cigarette smoke, pharmacological agents or lipopolysaccharides. We are using both types of model systems to study the effects of cigarette smoke extract on single and co-cultures composed of AT I cells and MVECL in order to better understand the role of AT I cells in the pathogenesis of emphysema.

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Figure 1. Markers of AT I and MVECL purity in cell preparations

(A) AT I cells stained for T1 α and (B) AQ-5 (inserts are respective negative controls); (C) AT I cells stained for vimentin (insert is positive control of human dermal foreskin fibroblasts) with companion (D) phase contrast and (E) DAPI images. (F) MVECLs stained for VE-cadherin. Bars = 50 μ m.

Figure 2. AT I cell proliferation

Live AT I cells at 1 day (A), 4 days (B), and 8 days (C) post seeding. Bars = 50 μ m. (D) Graph of data from the Cell Titer 96[®] AQ_{ueous} One Solution Cell Proliferation Assay, an MTS assay, showing an increase in relative AT I cell number over 96 hours. Student's t-test was used to determine if there was a significant increase in cell number from 24 hours to 96 hours at each PD ($p < 0.05$); $n = 3$, error bars are SE.

Figure 3. AT I cell characteristics

Staining of AT I cells to detect (A) mitochondria and (B) actin filament arrangement. Bars = 50 μm . (C) Relative telomere length in AT I cells at PD 6-7, 21-23, 30-35 and 100. Student's t-test was used to determine if there was a significant decrease in telomere length at PD 100 compared to PD 6-7 ($p < 0.05$); $n = 3$, error bars are SE.

Figure 5. Co-culture of AT I and MVECL

(A) DiI labeled AT I cells (red) seeded to the top of fibronectin coated 24-well Transwell filter; Bar = 100 μ m. (B) CFDA SE labeled MVECL (green) seeded to the bottom of filter. Bar = 100 μ m. Image inserts are higher magnification. (C) Electron micrograph of AT I cells grown on fibronectin coated 24-well Transwell filter insert (BM= basement membrane, C= caveolae, M= mitochondria, Mv= microvilli, PD = phagocytic debris). Bar = 500 nm. (D) Electron micrograph of tight junction (arrow) between two AT I cells. Bar = 100 nm.

Figure 6. T1α, AQ-5, ZO-1 expression in AT I and VE-cadherin expression in MVECL grown on fibronectin coated Transwell filter inserts

AT I cells grown on fibronectin coated Transwell filter express (A) T1α, (B) AQ-5, and (C) ZO-1. Arrow points to peripheral ZO-1 while purplish-pink staining of nuclei suggests co-localization with DAPI. Insert is a higher magnification view of ZO-1 at the cell border; Bars = 50 μm. (D) MVECLs grown on fibronectin coated Transwell filter express VE-cadherin; Bar = 20 μm.

Figure 7. AT I cells in thin and thick (3-dimensional) Matrigel

A) AT I cells 11 days post seeding on thin Matrigel; B) AT I cells 2 days post seeding into thick Matrigel, arrows point to elongated cells; C) AT I cells 8 days post seeding into thick Matrigel; D) AT I cells 11 days post seeding into thick Matrigel, arrows point to nuclei of ring-like structure; Nuclei (arrows) of cells in the ring-like structure stained with DAPI (insert); Bars= 50 μm.

Table 1

Morphometric characteristics of AT I cells.

Characteristic	PD 6-7				PD 20-24				PD 30-35			
	N	Mean	± S.E.	N	Mean	± S.E.	N	Mean	± S.E.	N	Mean	± S.E.
Cell Area	128	4978.7	341.9	113	5504.6	431.6	127	5636.2	487.6			
Cell Perimeter	128	320.6	10.7	113	464.8	43.8	127	389.4	6.1			
Senescent Cell Area	6	29084.2	5745.1	9	14739	2773.1	16	41561.4	9524.2			
Apoptotic Cells	120	1	0.05	120	1	0.065	120	2	0.104			
Mitochondrial Location												
0-10 μm from nucleus	120 ^a	2.40	0.134	120	2.51	0.121	120	2.57	0.138			
10-20 μm from nucleus	120 ^a	2.22	0.134	120	2.53	0.137	120	2.23	0.131			

N= number of cells counted;

^a = number of 10 μm^2 areas counted. One way ANOVA was used to assess for differences in morphometric characteristics of AT I cells at PD 6-7, 20-24 and 30-35. There was no difference in the measured morphometric characteristics between cells at PD 6-7, 20-24 or 30-35 indicating that AT I cells maintained their measured morphometric characteristics.